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# INTERACTION OF MELITTIN WITH DIMYRISTOYL PHOSPHATIDYLCHOLINE LIPOSOMES

## EVIDENCE FOR BOUNDARY LIPID BY RAMAN SPECTROSCOPY

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## Summary

The interaction of melittin, a polypeptide consisting of 26 amino acid residues, with dimyristoyl phosphatidylcholine bilayers was investigated by vibrational Raman spectroscopy. Spectral peak height intensity ratios, involving vibrational transitions in both the 3000 $cm^{-1}$ acyl chain methylene carbon-hydrogen stretching mode region and the 1100 $cm^{-1}$ acyl chain carbon-carbon skeletal stretching mode interval, served as temperature profile indices for monitoring the bilayer order-disorder processes. For a lipid : melittin molar ratio of 14 : 1 two order-disorder transitions were observed. In comparison to a gel to liquid crystalline phase transition of 22.5°C for the pure lipid, the lower transition, exhibiting a 2°C width, is centered at 17°C and is associated with a depression of the main lipid phase transition of dimyristoyl phosphatidylcholine. The second thermal transition, displaying a 7°C interval, occurs at approx. 29°C and is associated with the melting behavior of approximately seven immobilized boundary lipids which surround the inserted hydrophobic segment of the polypeptide. For a lipid : melittin molar ratio of 10 : 1 two thermal transitions are also observed at 11 and 30°C. As before, they represent, respectively, the main gel to liquid crystalline phase transition and the melting behavior of approximately four boundary lipids attached to melittin. From these data alternative schemes are suggested for disposing the immobilized lipids around the hydrophobic portion of the polypeptide within the bilayer.

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Abbreviation: DMPC, dimyristoyl phosphatidylcholine.

## Introduction

In describing the conformational and dynamic properties of lipids and proteins in reconstituted biological membranes, recent studies have suggested the existence of discrete lipid boundary regions surrounding the intruding bilayer components (see, for example, Refs. 1—6). Although a detailed understanding of the structural characteristics of the lipid components at the protein interface remains limited, the most useful physical approaches toward clarifying the nature of this immobilized boundary layer stem, at the present, from applications of electron spin resonance and nuclear magnetic resonance techniques [1,6—8]. In relating the derived molecular parameters from these experiments to membrane models, it is important to recognize that the various time scales probed by the individual procedures reflect different motional properties of the molecular assembly [8]. Thus, for example, fluctuations of the order of $1 \cdot 10^8$ $s^{-1}$ effect the ESR spectrum, whilst rates of motion greater than or equal to approx. $1 \cdot 10^5$ $s^{-1}$ are reflected by $^2$H-NMR spectra. In order to characterize further the effects of lipid-protein associations on both the intra- and intermolecular properties of particularly the lipid matrix, it is desirable to complement the magnetic resonance experiments with studies in which molecular events are monitored on the vibrational time scale in which atomic motions occur of the order of $1 \cdot 10^{13}$ $s^{-1}$. Raman light-scattering techniques and infrared absorption methods are capable of probing biological membranes within this time domain in a non-perturbative manner.

Although vibrational Raman spectroscopy provides an extremely sensitive means for monitoring specifically the conformational changes induced within the lipid matrix [9—16], relatively few studies employing vibrational techniques support the notion for a separate lipid environment surrounding an intrinsic membrane component [17—19]. For example, Raman spectra characteristic of the methylene C-H stretching modes within the lipid acyl chains were investigated by Verma and Wallach [17] in an assessment of the effect of melittin on the gel to fluid phase transitions of both sonicated dimyristoyl phosphatidylcholine (DMPC) and egg lecithin liposomes. These authors interpreted changes in the spectral data in terms of a lipid-polypeptide interaction that induced an increase in the gel-liquid crystalline phase transition [17]. In our discussion below we will present evidence that the phase transition temperature for this system in unsonicated liposomes does not increase, but on the contrary, is depressed. In a vibrational infrared study involving the effect of cholesterol-amphotericin B interactions on the melting behavior of lipid chains in DMPC single shell vesicles, it was noted that the sterol-antibiotic complexes ordered the gel phase bilayer [18]. Furthermore, the significantly broadened phase transition and the particularly higher temperature observed for the completion of the phase transition for antibiotic-containing vesicles, as compared to the observations for the pure liposomes, also suggest a population of immobilized lipid associated with the sterol-amphotericin B complex in the liquid crystalline state [18]. Recent Raman studies involving the perturbation of myelin proteolipid apoprotein on the thermal phase behavior of DMPC and egg lecithin multilayers also implied the existence of a class of protein-associated lipids [19]. Curatolo et al. [19] monitored the lipid phase behavior, however,

primarily by following intensity changes in the 2800—3000 $cm^{-1}$ acyl chain C-H stretching mode region. Only a limited amount of data on this system were obtained in the 1000—1150 $cm^{-1}$ area, the C-C stretching mode region [19]. This latter spectral interval is important in classifying bilayer systems as it reflects the extent of *trans/grauche* isomerization arising along the lipid chains.

In this communication we further examine the utility of Raman spectral data for detecting the presence of an immobilized lipid annulus about an intrinsic membrane constituent. The bilayer system consists of DMPC liposomes incubated with melittin, a small protein (consisting of 26 amino acid residues) capable of spontaneously integrating itself within the membrane. In particular, we examine temperature profiles based upon vibrational data characteristic of both intermolecular and intramolecular order/disorder parameters of the acyl chains comprising the lipid matrix. From these data and assumptions concerning the conformation of melittin within the bilayer, alternative schemes for disposing the immobilized lipids about the hydrophobic portion of the polypeptide are suggested.

## Experimental Procedure

High-purity samples of 1,2-dimyristoyl-L-α-phosphatidylcholine were obtained commercially from Sigma Chemical Company. Melittin was prepared from whole bee venom as previously described [20].

Melittin was added to aqueous dispersions of DMPC (approx. 15% by weight) to yield final lipid : protein ratios of 10 : 1 and 14 : 1. These mixtures were first mechanically shaken for 10 min and then kept at 40°C for 1 h to allow the peptide to interact completely with the DMPC multilayers. The melittin-containing liposomes were transferred to 1.5—1.8-mm diameter glass capillaries for obtaining Raman spectra. The concentrated 10 : 1 mixtures were clear, rather than turbid, reflecting a posible breakdown of the large multilamellar structures into smaller bilayer units.

Vibrational Raman spectra were recorded with a Spex Ramalog 6 spectrometer equipped with plane holographic gratings. A Coherent Model Cr-3 argon ion laser served as the excitation source, which at 514.5 nm typically provided 100 mW at the sample. The spectral resolution was of the order of 5 $cm^{-1}$; spectral frequencies, calibrated with atomic argon lines, are reported to ±2 $cm^{-1}$. Capillaries containing the samples were placed within a holder which was thermostatically controlled to ±0.05°C. Temperatures were monitored by a copper-constantan thermocouple inserted into the capillary near the laser beam transit. In determining heating curves for the lipid dispersions, the experiments progressed from low to high temperatures. After equilibrating the samples at each temperature for approx. 15 min, Raman spectra were acquired using a Nicolet NIC-1180 data system interfaced to the spectrometer. The 2800—3000 $cm^{-1}$ C-H and the 990—1200 $cm^{-1}$ C-C stretching regions, respectively, were scanned at a rate of 1 $cm^{-1}$/s using signal-averaging techniques. Six scans were required for the 990—1200 $cm^{-1}$ interval for the 14 : 1 dispersions, whilst three scans were sufficient for the 2800—3000 $cm^{-1}$ region. A slightly greater number of scans (8—10) were required in scanning each spectral region for the 10 : 1 samples. Temperature profiles were constructed from original, unsmoothed spectral

from peak height intensity ratios for the $I_{2940}/I_{2885}$ interchain disorder-order parameters and the $I_{1090}/I_{1130}$ intramolecular *gauche-trans* isomerization parameters.

The number of lipid molecules involved in the boundary layer was estimated by assuming that the entire melting curve, composed first of the main phase transition and, second, the melting of the immobilized lipid, reflects a linear behavior in the fluidization of the lipid matrix.

## Results and Discussion

Fig. 1 and 2 display representative spectra for the C-C and C-H stretching mode regions, respectively, for the lipid hydrocarbon chains. For the gel phase of DMPC at 12.5°C, Fig. 1 compares spectra for a pure DMPC bilayer and a DMPC bilayer containing melittin in a 10 : 1 lipid : protein mole ratio. The observed intensities for the vibrational transitions assigned to C-C stretching modes at 1126, 1088 and 1061 $cm^{-1}$ in pure DMPC bilayers imply that the hydrocarbon chains possess a high degree of intramolecular order, exhibiting perhaps of the order of one *gauche* bond per chain at the methyl terminus [9,10]. At the same temperature, however, melittin further disorders the chains as evidenced by the frequency shifts to 1124 and 1083 $cm^{-1}$ and the change in relative intensities of the three primary features [10,21]: (mellitin does not contribute vibrational transitions to this spectral area; consequently no spectral subtraction is required for the C-C stretching region). In addition, for the disordered bilayers containing melittin, a shoulder, which does not appear in disordered pure DMPC, occurs at approx. 1088 $cm^{-1}$. Furthermore, the 1124 $cm^{-1}$ feature, compared to the 1126 $cm^{-1}$ vibrational transition for pure DMPC, is broadened slightly. These observations imply the presence of small populations of more ordered lipids within the generally perturbed bilayer.

Fig. 2 presents comparisons of pure DMPC and melittin-containing bilayers

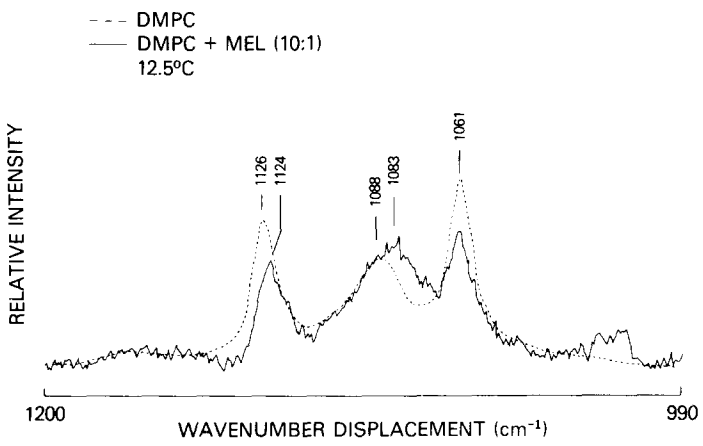


Fig. 1. Raman spectra of DMPC-melittin liposomes in the 1100 $cm^{-1}$ region at 12.5°C. (———) System composed of a 10 : 1 DMPC : melittin mole ratio; (- - - - -) pure DMPC bilayer assembly. MEL, melittin.

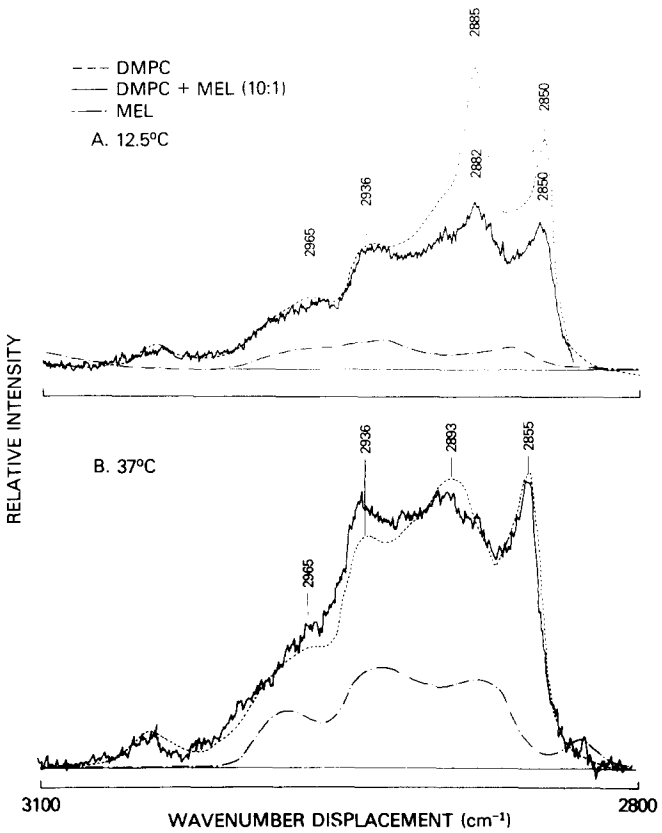


Fig. 2. Raman spectra of DMPC-melittin liposomes in the 3000 $cm^{-1}$ region for (A) the gel state at 12.5°C and (B) the liquid crystalline state at 37°C. (———) System composed of a 10 : 1 DMPC : melittin mole ratio; (- - - - -) pure DMPC bilayer assembly. (— · — ·) Normalized contribution of melittin to the lipid plus melittin (MEL) spectra.

for the C-H stretching modes in both the gel and liquid crystalline states. Since high polypeptide concentrations were used in these experiments, spectral contributions from melittin in the 3000 $cm^{-1}$ range must be subtracted before determining temperature profiles based upon peak height intensity ratios (vide infra). The required background spectra used in the subtraction procedure were obtained from appropriate melittin/water mixtures that were also scanned as a function of temperature. An increase in intensity of the melittin features within the C-H stretching region occurred at temperatures below approx. 10°C, perhaps implying the dominance of the tetrameric form under these conditions in water [22]. No correction for a tetramer $\rightleftharpoons$ monomer equilibrium, which is present in aqueous solution, was made in performing the spectral subtractions from the bilayer spectra. For the liquid crystalline state of the melittin-DMPC bilayers, Fig. 2B, a shoulder appears at 2885 $cm^{-1}$. This vibrational features correlates with the methylene C-H antisymmetric stretching modes at 2885 $cm^{-1}$ for the ordered lipid conformation (see Fig. 2A) and may suggest the existence of ordered domains in the liquid crystalline phase. It is also possible that the 2885 $cm^{-1}$ feature in the liquid crystalline phase arises in part from the under-

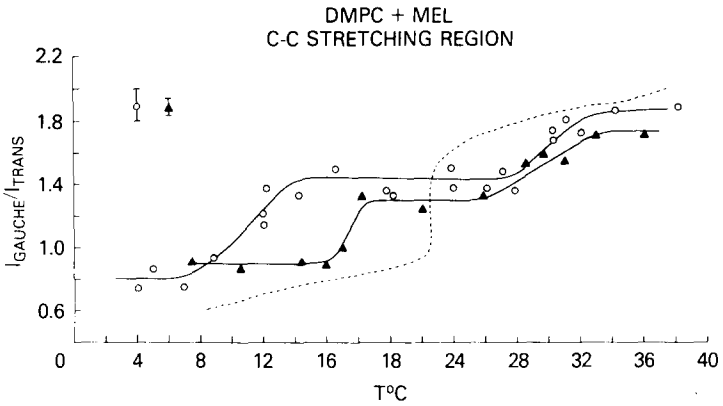


Fig. 3. Temperature profiles for DMPC-melittin liposomes using the $I_{1090}/I_{1130}$ ($I_{gauche}/I_{trans}$) peak height intensity ratios as markers. (▲) Profile for a 14 : 1 DMPC : melittin mole ratio system; (○) profile for a 10 : 1 DMPC : melittin mole ratio system. (- - - - -) Pure DMPC bilayer assembly.

lying C-H stretching modes of melittin that are not completely accounted for in the subtraction procedure. Further elaboration of this point must await our current studies, involving deuterated lipid chains, in which we explicitly distinguish between the lipid and polypeptide C-H stretching modes.

Temperature profiles for the hydrocarbon chain C-C stretching region, Fig. 3, were formed from the ratios $I_{gauche}/I_{trans}$, where $I_{gauche}$ and $I_{trans}$ represent peak height intensities for the 1090—1080 and 1126 $cm^{-1}$ features, respectively. An increase for this ratio reflects an increase in chain disorder through the introduction of *gauche* bonds [9,10]. For the 14 : 1 lipid-polypeptide mole ratio two order-disorder transitions are observed. The lower transition, centered at 17°C, is associated with a depression of the main lipid phase transition of DMPC, which occurs at 22.5°C for the pure system. The 17°C transition is broadened by approx. 2°C, compared to the sharp DMPC transition of approx. 0.5°C, or less: (for clarity in Figs. 3 and 4, the experimental points for the profile for pure DMPC are not placed in the graph). The second thermal transition, centered at approx. 29°C, displays a 7°C transition interval. We associate this higher order-disorder transition with a fluidization of the immobilized boundary lipids present at the polypeptide/lipid interface within the bilayer. For this peptide concentration the temperature profile indicates that approximately seven lipids are associated with the melting process of the boundary lipid.

Fig. 3 also presents a temperature profile, based upon the C-C acyl chain stretching modes, for a 10 : 1 mole ratio for the lipid-polypeptide system. As before, two thermal order-disorder transitions are observed. The lower transition at 11°C is again associated with a depression of the main DPMC gel to fluid phase transition; whilst the higher transition at 30°C represents the melting behavior of the boundary lipid about melittin. For this lipid-polypeptide mole ratio the broadened primary and boundary layer transitions display 7 and 5°C transition widths, respectively. The increase in breadth of the lower transition, compared to the 14 : 1 mixture, may be a result of the formation of smaller liposomal structures caused by high protein concentrations. For this system,

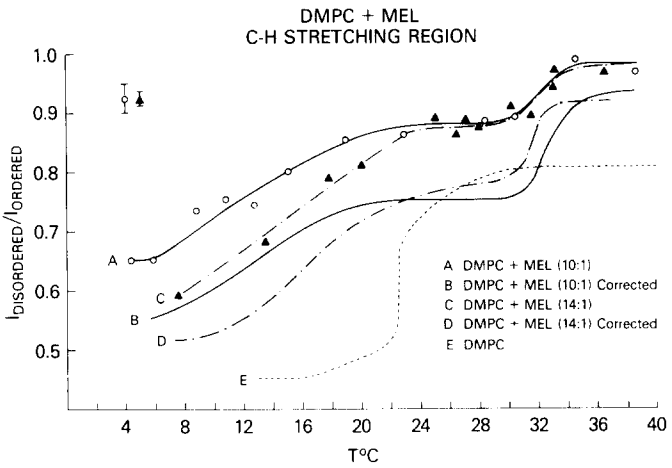


Fig. 4. Temperature profiles for DMPC-melittin liposomes using $I_{2940}/I_{2880}$ ($I_{disordered}/I_{ordered}$) peak height intensity ratios as markers. Curves A and C represent the observed profiles for the 10 : 1 and 14 : 1 DMPC : melittin mole ratio systems, respectively. Curves B and D represent the 10 : 1 and 14 : 1 DMPC : melittin profiles, respectively, corrected for spectral contributions from melittin C-H stretching mode vibrations. Curve E represents the pure DMPC bilayer assembly. MEL, melittin.

containing the higher peptide molar concentration, approximately four lipids appear to be immobilized by the hydrophobic region of melittin.

Fig. 4 displays the temperature profiles based on the peak height intensities for the methylene $CH_2$ antisymmetric stretching modes at 2940 and 2880 $cm^{-1}$. Increase in intensity of these two vibrational transitions reflects intermolecular chain disorder and order, respectively [10]. The ratio $I_{2940}/I_{2880}$ is represented in Fig. 4 by the quantity $I_{disordered}/I_{ordered}$. Since the vibrational spectrum of melittin overlaps the C-H stretching mode region of DMPC for the high concentrations of peptide used in these lipid dispersions, we also display corrected profiles for which the peptide emission has been subtracted.

As demonstrated by the temperature profiles displayed in Fig. 4, the polypeptide induces significant lattice disorder into the lipid gel phase. The primary order-disorder transitions are centered at approx. 12 and 16°C for the 10 : 1 and 14 : 1 lipid-polypeptide mode ratios, respectively, which is in good agreement with the data from the C-C stretching mode regions for these systems. The transitions determined by the C-H parameters are significantly broadened (11 and 9°C intervals for the 10 : 1 and 14 : 1 systems, respectively) compared to their counterparts derived from the C-C spectral regions. These differences originate in the use of different parameters for reflecting either the inter- or intramolecular behavior of the lipid bilayer. The second thermal phase transition, representative of the fluidization of the immobilized lipid about the peptide appears at approx. 32—33°C and suggests that approximately five lipids are involved in this specific disordering process for both concentrations of polypeptide. Fig. 4 also indicates that at high temperatures (approx. 38°C) the bilayer lattice structure is significantly more disordered than that for pure DMPC in its liquid crystalline state.

Using different C-H stretching mode intensity ratios and different lipid-

protein ratios than those in the present study, Verma and Wallach [17] observed only the higher transition in their temperature profiles. They associated the higher temperature, as compared to a pure lipid system, to an increase in the main gel-liquid crystalline phase transition, rather than to a melting of the boundary layer as presented here.

As noted above, the 2885 $cm^{-1}$ feature observed in the liquid crystalline phase of the DMPC-melittin system (Fig. 2B) may suggest the existence, after the melting behavior of the boundary lipids, of a population of ordered or perturbed lipids which assume conformations other than those found in the bulk lipid matrix. For DMPC liposomes containing myelin proteolipid apoprotein, Curatolo et al. [19] also associate a feature at approx. 2885 $cm^{-1}$ with unmelted DMPC chains. Evidence for ordered lipids, relative to pure DMPC multilayers, after the fluidization of the boundary lipids is noted in the temperature profiles for the C-C stretching modes displayed in Fig. 3. Thus, the high temperature point at approx. 36°C for the 14 : 1 lipid-polypeptide system represents more ordered acyl chains than those for pure DMPC. The more concentrated 10 : 1 system also suggests slightly more order than pure DMPC; but from the errors associated with a determination of the temperature profile, one could argue that the 10 : 1 melittin-containing multilayers possess approximately the same disorder present in the pure bilayers. Although we cannot eliminate the possibility of a change in protein conformation between 28 and 32°C, the lower position of the plateau in the temperature profile for the C-C stretching mode region, compared to pure DMPC, (Fig. 3) and the presence of the 2885 $cm^{-1}$ feature in the liquid crystalline spectra of melittin-DMPC system, strongly indicates that the dominant effect involves a further fluidization of lipids.

The estimate for the involvement of approximately seven lipids in the boundary layer around melittin for the 14 : 1 DMPC : polypeptide multilayers is consistent with a value of six bound lipid molecules for a 25 : 1 system (Lavialle, F., Levin, I.W. and Mollay, C., unpublished observations). A value of approximately four lipids for the 10 : 1 liposomes suggests that the lipid-lipid interactions are stronger than the lipid-polypeptide associations and that perhaps approximately six lipids are necessary for stabilizing the bulk bilayer matrix between the intrinsic polypeptide units. It is also possible, however, that for this more concentrated lipid : polypeptide system, peptide-peptide interactions may become significant [23].

Although the conformation of melittin with a phospholipid bilayer has not been definitely established, both NMR studies involving melittin-micelle interactions [24] and fluorescence studies concerned with melittin-bilayer interactions [25,26] support the notion that the predominantly hydrophobic region of the polypeptide, residues 1—19, penetrates the hydrophobic interior of the bilayer, while the polar portion, residues 20—26, remain exterior to the bilayer. These conclusions are supported by Raman spectroscopic studies involving interactions of the separate melittin hydrophobic (1—19) and hydrophilic (20—26) fragments with lipid bilayer systems (Lavialle, F., Levin, I.W. and Mollay, C., unpublished observations). Despite the limited conformational knowledge regarding the hydrophobic segment of melittin, it is interesting to speculate upon molecular models for the peptide that would be consistent with

approximately six or seven bilayer lipids bound to be peptide interface. Since melittin is predicted to be in an α-helical form in a lipid environment [27], we may roughly estimate for this type of structure the number of bilayer lipids constituting the primary annulus around the peptide as follows. Although proline-14 probably leads to the formation of two helical regions for the hydrophobic segment, we shall consider that the first nineteen residues form a cylinder of which the height is approx. 28.5 Å [28]. A specific volume of 0.72 $cm^3/g$ [29] for the polypeptide yields an estimate for the diameter of the cylinder of approx. 10.1 Å. If we assume that the helical hydrophobic segment spans the acyl chain portion of the bilayer, then we are able to approximate the number of lipid molecules that could be fit around the polypeptide. Thus, for a surface radius of 4.4 Å for a DMPC molecule in a liquid crystalline bilayer 35 Å thick [30], each monolayer of the bulk lipid matrix would contribute approximately seven lipids to the first boundary shell about the peptide cylinder; that is, a total of fourteen lipids would be expected for the interfacial layer.

Since a total of approximately five to seven associated lipids, rather than approximately fourteen, were determined from the temperature profiles, several possibilities are envisioned for distributing the boundary lipids about the polypeptide. (1) For a model in which the predominantly hydrophobic portion of the amino acid sequence, residues 1—19, spans the bilayer, only the monolayer surrounding the helix to lysine-7 (the first basic amino acid from the N-terminus) contributes strongly associated lipids to the peptide. In addition to lysine at the seventh residue, threonine-10, threonine-11 and serine-18, which contain uncharged polar groups, would tend to reduce substantially hydrophobic interactions with lipids defining the monolayer of which the head groups electrostatistically bind the hydrophilic segment of melittin. (2) Since molecular models suggest that the amino acids containing both basic and uncharged polar groups, lysine-7, theonine-10 and threonine-11, are oriented to one face of the inserted helix [27], both monolayers could contribute a total of approximately seven lipids to the boundary layer; however, strong associations would occur only with the hydrophobic areas of the polypeptide. (3) Lysine-7, a basic amino acid may prefer to orient itself near the aqueous phase at the interface region between the head group and acyl chain regions of the bilayer. Thus, only one monolayer would contribute toward a boundary layer for the short helical region composed to residues 1—6. The remainder of the generally hydrophobic fragment to residue 19 may then extend through the bilayer region perhaps as a random coil. A conformation of this nature for this portion of the sequence would probably not result in a strongly associated boundary region of lipid.

In summary, vibrational Raman spectra data provide evidence for a class of immobilized lipids associated with the interface of the hydrophobic segment of melittin, an intrinsic membrane constituent. The portion of the temperature profiles identified with the boundary lipids exhibit a broad melting behavior at temperatures substantially above the gel-liquid crystalline phase transition of the bulk lipids. Temperature profiles determined from spectral intensity changes in both the lipid acyl chain C-H and C-C stretching mode regions indicate approximately five to seven lipids form the initial boundary layer around the polypeptide.

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